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Oscillations of Membrane Potential in L Cells

IV. Role of Intracellular Ca^{2+} in Hyperpolarizing Excitability

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Summary. Effects of divalent cations on oscillations of membrane potentials (i.e., spontaneous repetitive hyperpolarizing responses) and on hyperpolarizing responses induced by electrical stimuli as well as on resting potentials were studied in large nondividing L cells. Deprivation of Ca^{2+} from the external medium inhibited these hyperpolarizing responses accompanying slight depolarization of the resting potential. Sr^{2+} or Mn^{2+} applied to the external medium in place of Ca^{2+} was able to substitute for Ca^{2+} in the generation of hyperpolarizing responses, while Mg^{2+}, Ba^{2+} or La^{3+} suppressed hyperpolarizing responses. The addition of A23187 to the bathing medium or intracellular injection of Ca^{2+}, Sr^{2+}, Mn^{2+} or La^{3+} induced membrane hyperpolarization. When the external Ca^{2+}, Sr^{2+} or Mn^{2+} concentration was increased, the resting potential also hyperpolarized, in a saturating manner. The amplitude of maximum hyperpolarization produced by high external Ca^{2+} was of the same order of magnitude as those of hyperpolarizing responses and was dependent on the external K^+ concentration. In the light of these experimental observations, it was deduced that the K^+ conductance increase associated with the hyperpolarizing excitation is the result of an increase in the intracellular concentration of free Ca^{2+} mainly derived from the external solution.

Fibroblastic L cells in culture were found not only to respond with a transient hyperpolarization to a mechanical or electrical stimulus (Nelson, Peacock & Minna, 1972) but also to show spontaneous, repetitive hyperpolarizations (i.e., oscillations of the membrane potential) under a controlled condition (Okada *et al.*, 1977*a*). We postulated that such oscillating hyperpolarizing responses occurred spontaneously (spontaneous HRs) and hyperpolarizing responses evoked by stimuli (evoked HRs) are caused solely by an increase in K^+ conductance across the cell membrane (Okada *et al.*, 1977*b*; Roy & Okada, 1978), and are related to cellular metabolic activities (Okada *et al.*, 1977*a*).

Similar hyperpolarizing excitability of cell membranes has been recently found in the other cells including macrophages (Gallin *et al.*, 1975; Dos Reis & Oliveira-Castro, 1977), adenohypophysis cells (Poulsen & Williams, 1976), sympathetic ganglion cells (Kuba & Nishi, 1976), mega-

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karyocytes (Miller, Sheridan & White, 1978), and cultured syncytiotrophoblasts of human placenta (J. Yano, W. Tsuchiya & Y. Okada, *unpublished observation*). Although the physiological function(s) of these hyperpolarizing responses (HRs) has not been determined precisely, it was suggested that such an HR of the cell membrane may mediate important cell activities (Nelson & Peacock, 1973; Gallin & Gallin, 1977; Tsuchiya, Okada & Inouye, 1978). Acquisition of data on the fine concept of ionic mechanism for HRs should throw additional light on the physiological meaning of HR.

Based on observations using chelating agents and a divalent cation ionophore (A23187), Gallin *et al.* (1975) and Kuba and Nishi (1976) suggested that the intracellular free Ca^{2+} might regulate those HRs by affecting the membrane K^+ conductance of macrophages and sympathetic ganglion cells treated with caffeine, respectively. We have obtained further evidence of this concept in L cells and have concluded that intracellular Ca ions are regulating the HR activity, and it was suggested that these cations are derived mainly from outside the cell but not from some intracellular organella, as described herein.

Preliminary results have been presented in an abstract form (Okada, Tsuchiya & Inouye, 1978).

Materials and Methods

The cell cultures and electronic apparatus employed herein have already been reported by Okada *et al.* (1977*a*).

A monolayer of large nondividing L cells obtained by X-ray irradiation was used. To obtain stable oscillations of membrane potentials from almost all the cells, culture media in glass petri dishes were replaced by fresh media every other day and electrophysiological measurements were performed on the day after the medium renewal. Fiber filled glass microelectrodes (Tasaki *et al.*, 1968; Okada & Inouye, 1976) were employed for recordings, and the resistance and tip potential ranged from 15 to 30 MΩ and from 0 to −5 mV, respectively. Divalent or trivalent cations (Ca^{2+}, Mg^{2+}, Ba^{2+}, Mn^{2+}, Sr^{2+} or La^{3+}) were injected into the cell from a microelectrode filled with 3 M KCl and 0.1 M chloride salts of these cations by the outward (depolarizing) current through a bridge circuit (WPI-M701). As a rule, we applied iontophoretic currents of 10 nA for periods of up to 3 sec; even using such moderate currents, micropipettes filled with divalent or trivalent cations became blocked very quickly inside the cell. To minimize such a blocking process, electrodes of relatively low resistance (8 ~ 10 MΩ) were selected. Sometimes, a tip of the electrode with a high resistance was polished by inserting it into tooth paste several times, as described previously (Okada & Inouye, 1978). This procedure was found to be useful in reducing the electrode resistance as well as the tip potential without introducing any artifacts in the electrical recordings. Injection of chelating agents into cells was carried out using 3 M KCl–0.1 M EDTA (or potassium citrate) by applying an inward current. The pH of

KCl-EDTA solution was adjusted to 6.3 with tris(hydroxymethyl)-aminomethane (Tris) and HCl; at this pH, almost all the EDTA is negatively charged (Carini & Martell, 1952).

The Tris-buffered saline (TBS) was used as a control medium and was composed of 143.0 mM NaCl, 4.2 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 20 mM mannitol and buffered to pH 7.3 ± 0.1 with 10 mM Tris-HCl. Changes in K^+ concentrations were accomplished by replacing all or a part of NaCl with equimolar amounts of KCl under the fixed sum of K^+ and Na^+ concentrations (147.2 mM). Low-Na^+ solutions (1 mM Na^+) were prepared by replacing 142.0 mM NaCl in the control TBS with 142.0 mM LiCl or Tris-HCl. To obtain a high-Ca^{2+} or high-Mg^{2+} medium, an appropriate amount of $CaCl_2$ or $MgCl_2$ was added to TBS. As required, the entire amounts of $CaCl_2$ and/or $MgCl_2$ in TBS were deleted to obtain a Ca^{2+} and Mg^{2+}-free TBS, Ca^{2+}-free TBS or Mg^{2+}-free TBS. When the effect of divalent or trivalent cations was examined, various amounts of $SrCl_2$ or $MnCl_2$ were added to a Ca^{2+}-free TBS in the presence of 0.5 mM Mg^{2+}, and $BaCl_2$ or $LaCl_3$ to a Mg^{2+}-free TBS in the presence of 0.9 mM Ca^{2+}.

EDTA (Na_2-salt) and EGTA (Na_2Mg-salt) were employed as chelating agents. Ruthenium red was purchased from Chroma-Gesellschaft Schmidt & Co. The divalent cation ionophore, A23187, was a gift from Eli Lilly. Ethanol was used as a vehicle for the drug. The addition of up to 2% ethanol did not affect the electrical properties of cell membranes.

Control experiments were performed at 35 ± 2 °C, but the temperature of the cells was reduced to 6 ± 2 °C by circulating ice-cold water when necessary.

All the data presented herein are the means $\pm$ SE with the number of observations, *n*, in the parentheses.

Results

Effect of Deprivation of External Ca^{2+}

In the control medium, the membrane of L cell showed spontaneous oscillating hyperpolarizations (spontaneous HRs) as well as hyperpolarizing responses evoked by electrical stimuli (evoked HRs), as described previously (Okada *et al.*, 1977*a*). To study the effect of divalent cations on these hyperpolarizing responses (HRs) of the membrane, Ca^{2+} and Mg^{2+} were first removed from the external solution. As shown in Table 1, removal of both external Ca^{2+} and Mg^{2+} completely inhibited not only spontaneous HRs, but also evoked HRs, and slightly depolarized the resting potential. With the addition of EDTA (3 mM) in the Ca^{2+} and Mg^{2+}-free TBS, these HRs immediately disappeared and a significant depolarization of the resting potential occurred. Such effects of Ca^{2+} and Mg^{2+}-free conditions were reversible.

In the presence of external Mg^{2+}, the addition of EGTA (1 mM) to the external Ca^{2+}-free TBS also caused the suppression of HRs and a depolarization of the resting potential, as shown in Fig. 1 and Table 1. On the other hand, the deprivation of external Mg^{2+} in the presence

Table 1. Effect of Ca^{2+}-free conditions on the membrane potential in L cells

Condition	Resting potential (mV)	Spontaneous HR (mV)	Evoked HR (mV)
Control	-14.6 ± 0.5 (40)	-39.7 ± 1.6 (40)[d]	-38.4 ± 3.1 (8)
Ca & Mg-free[a]	-12.5 ± 0.4 (18)	–	–
EDTA[b]	-8.5 ± 0.6 (18)	–	–
Mg-EGTA[c]	-11.6 ± 0.5 (17)	–	–
Ca-free (total)	-10.9 ± 0.4 (53)	–	–

[a] 3 ~ 16 min after deprivation of whole amounts of $CaCl_2$ and $MgCl_2$ from a control TBS (Ca^{2+} and Mg^{2+}-free TBS).

[b] 2 ~ 13 min after addition of 3 mM EDTA to a Ca^{2+} and Mg^{2+}-free TBS.

[c] 2 ~ 14 min after addition of 1 mM EGTA to a Ca^{2+}-free TBS in the presence of 0.5 mM Mg^{2+}.

[d] The frequency of these oscillations was 3.9 ± 0.2 (40) cycle/min.

These values obtained under various Ca^{2+}-free conditions are significantly different from the control value at the < 5% level.

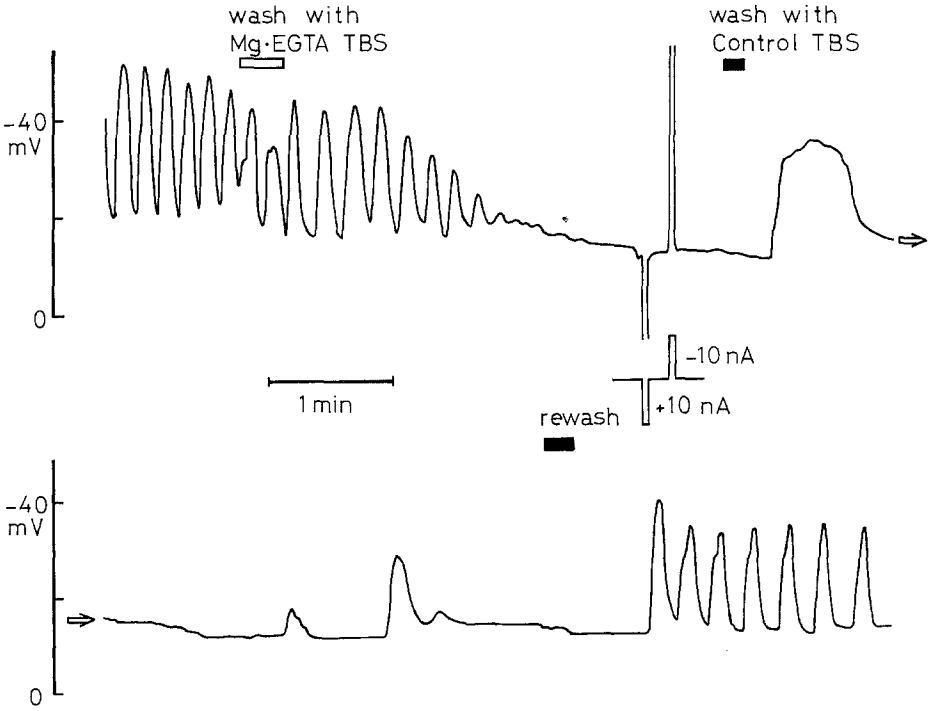

Fig. 1. Effect of Mg-EGTA on HR activities of L cells. The external media were changed during impalement of a recording microelectrode from a control TBS to a Ca^{2+}-free TBS containing 1 mM EGTA and 0.5 mM Mg^{2+}, and *vice versa*. Electrical currents (± 10 nA) were applied in an attempt to produce evoked HRs

of external Ca^{2+} did not remarkably affect the electrical properties of cell membranes. Therefore, it appears that the presence of Ca^{2+} (but not of Mg^{2+}) in the external medium is indispensable for the HR activities, what we term "hyperpolarizing excitability," of L cells. This result is in good agreement with observations made by Gallin *et al.* (1975) in macrophages and by Kuba and Nishi (1976) in sympathetic ganglion cells treated with caffeine.

Effect of A23187

Thus, the presence of external Ca^{2+} is essential for the production of membrane HR activity. Deprivation of external Ca^{2+} may in effect lower the internal free Ca^{2+} concentration of L cells by eliminating the leak or transport of external calcium into the cell. For clarification, we first used the carboxylic antibiotic A23187 which acts as a mobile carrier and transfers Ca^{2+} across cell membranes (Reed & Lardy, 1972; Pressman, 1973) and has been shown to increase cytoplasmic Ca^{2+} concentrations (Reed & Lardy, 1972; Foreman, Mongar & Gomperts, 1973; Steinhardt & Epel, 1974). Within a few seconds after addition of A23187 to the bath at the final concentration of $1 \sim 2$ μg/ml, the cell membrane underwent a prolonged hyperpolarization and a decrement in membrane resistance, and a higher dose of the ionophore ($4 \sim 10$ μg/ml) induced an even more prolonged hyperpolarization, as shown in Fig. 2*A*. At the same time, the oscillation was completely inhibited by the application of A23187, and the membrane resistance gradually decreased, as seen in Fig. 2*A*. It is possible that this antibiotic acts as a metabolic inhibitor as does valinomycin (Okada *et al.*, 1977*b*). The level of the peak hyperpolarization thus induced was tabulated in Table 2 and was somewhat lower than in the case of HRs (Table 2). Similar hyperpolarization has been obtained with A23187 in macrophages (Gallin *et al.*, 1975). This result suggests that an increase in the intracellular Ca^{2+} concentration in L cells resulted in hyperpolarization and that intracellular Ca^{2+} ions are responsible for the generation of hyperpolarizing excitability of these cells.

Effect of Intracellular Injection of Ca^{2+} or Other Ions

For direct verification, the effect of intracellular injection of Ca ions was observed. When a microelectrode filled with 3 M KCl and 0.1 M

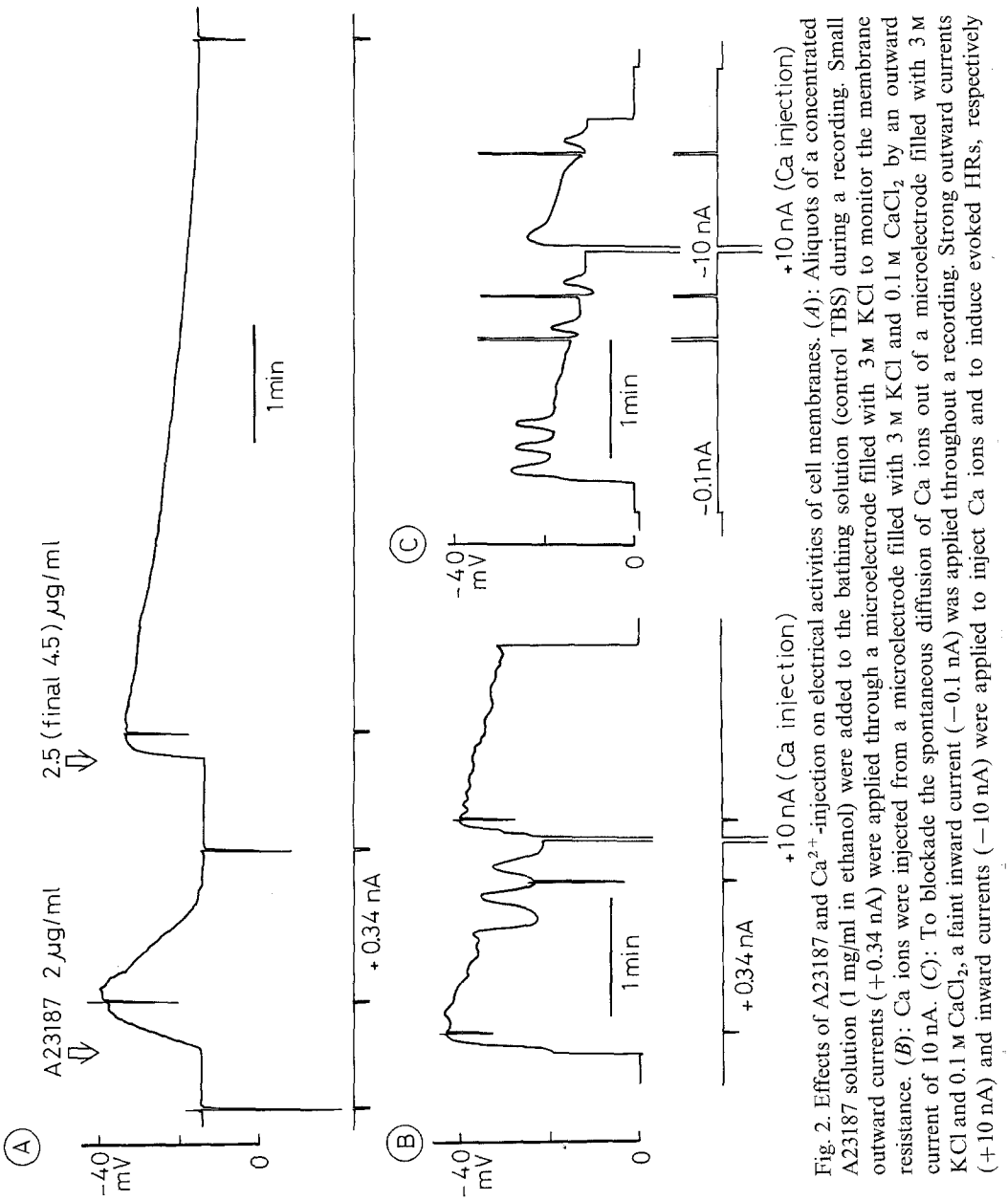

Fig. 2. Effects of A23187 and Ca^{2+}-injection on electrical activities of cell membranes. (*A*): Aliquots of a concentrated A23187 solution (1 mg/ml in ethanol) were added to the bathing solution (control TBS) during a recording. Small outward currents (+0.34 nA) were applied through a microelectrode filled with 3 M KCl to monitor the membrane resistance. (*B*): Ca ions were injected from a microelectrode filled with 3 M KCl and 0.1 M $CaCl_2$ by an outward current of 10 nA. (*C*): To blockade the spontaneous diffusion of Ca ions out of a microelectrode filled with 3 M KCl and 0.1 M $CaCl_2$, a faint inward current (−0.1 nA) was applied throughout a recording. Strong outward currents (+10 nA) and inward currents (−10 nA) were applied to inject Ca ions and to induce evoked HRs, respectively

$CaCl_2$ was inserted into the cell, the membrane potential thus measured remarkably hyperpolarized after the initial, short-lasting resting potential as illustrated in Fig. 2*B*. This prolonged hyperpolarization after an insertion of the microelectrode seems to be induced by Ca^{2+} ions diffused out of the micropipette. After such a long-lasting hyperpolarization,

Table 2. Hyperpolarizations induced by A23187 or intracellular injections of divalent or trivalent cations

	Peak potential (mV)	Significance of difference (P)
Spontaneous HR	-39.2 ± 1.9 (27)	–
Evoked HR	-40.4 ± 2.1 (26)	> 0.5
A23187 ($1 \sim 10$ μg/ml)	-35.6 ± 1.1 (9)	> 0.05
Ca^{2+}-injection	-40.2 ± 2.3 (14)	> 0.5
Sr^{2+}-injection	-42.7 ± 2.8 (10)	> 0.25
Mn^{2+}-injection	-41.0 ± 3.6 (9)	> 0.5
La^{3+}-injection	-42.7 ± 1.8 (6)	> 0.05

in some cases, the potential returned to the resting level and initiated oscillations as seen in Fig. 2*B*. A strong depolarizing current applied to the cell to inject Ca^{2+} during the oscillation again induced a prolonged hyperpolarization (Fig. 2*B*). Membrane resistances on such hyperpolarizations were less than those at the resting state. When a constant braking current (a hyperpolarizing current of 0.1 nA) was applied during a recording to prevent a diffusion of Ca ions from the micropipette, typical oscillations were often observed without initial prolonged hyperpolarizations as shown in Fig. 2*C*. In this case, a strong depolarizing current induced a prolonged hyperpolarization, while a strong hyperpolarizing current induced only a transient hyperpolarizing response (Fig. 2*C*). These observations indicate that the prolonged hyperpolarization obtained either after an insertion of a Ca^{2+}-containing micropipette or after the electrophoresis of Ca^{2+} was the result of an increase in the intracellular Ca concentration. The level of peak hyperpolarizations induced by Ca^{2+} injection was of the same order of magnitude as that induced spontaneously or electrically (Table 2).

There is a growing body of evidence that a rise in the intracellular Ca^{2+} concentration leads to an increased membrane permeability to K^+ in a variety of cells, including red blood cells (Gardos, 1958; Hoffman, 1966; Whittam, 1968; Lew, 1970; Riordan & Passow, 1971; Romero & Whittam, 1971; Blum & Hoffman, 1971, 1972), liver cells (van Rossum, 1970), and neurones (Godfraind, Krnjević & Pumain, 1970; Godfraind *et al.*, 1971; Meech, 1972; Jansen & Nicholls, 1973; Gorman & Marmor, 1974). This idea of Ca^{2+}-activated K^+ channel was supported by the finding that intracellular injection of Ca^{2+} greatly increased the membrane K^+ conductance in neurones (Meech & Strumwasser, 1970; Krnjević & Lisiewicz, 1972; Meech, 1972, 1974; Brown &

Brown, 1973), and cardiac Purkinje cells (Isenberg, 1975). These data together with our findings with Ca^{2+}-injection suggest that the mechanism that underlies the generation of hyperpolarizing responses either occurring spontaneously or being evoked electrically is a rise in the internal Ca^{2+} concentration which, in turn, triggers an opening of K^+ gates or an activation of K^+ carriers (Roy & Okada, 1978) within the membrane of L cell.

Using the same electrophoretic technique, the other cations were injected into the L cell. Sr^{2+}, Mn^{2+} and La^{3+} injected also induced remarkable hyperpolarizations of the same order of magnitude as those induced by Ca^{2+} injection (Table 2), whereas Mg^{2+} and Ba^{2+} did not. Intracellular injection of Mg^{2+} or Ba^{2+} inhibited the oscillation yet maintained the ability to produce HR evoked by an electrical stimulus, as shown in Figs. 3*A* and *B*. Thus, intracellular Ca^{2+} as a trigger for the K^+ channel of the membrane can be substituted for by intracellular Sr^{2+}, Mn^{2+} or La^{3+}, but not by Mg^{2+} or Ba^{2+}. Intracellular EDTA-injection also inhibited the oscillation of L cell, yet an electric current still produced an evoked HR, as seen in Fig. 3*C*. The result of injection of citrate was also quite similar (data not shown).

Effect of External Concentration Changes of Ca^{2+} or Other Cations

On replacing the control bathing medium (TBS) with a high (3 mM) Ca^{2+} medium, the resting membrane potential remarkably hyperpolarized while preserving the HR activity, as shown in Fig. 4*A*. The greater the external Ca^{2+} concentration ($[Ca]_o$), the more negative was level of the resting potential, and saturation occurred at the external Ca^{2+} concentration above 10 mM. After attaining the saturation of hyperpolarized resting potential, HRs did not occur spontaneously and were not induced electrically, as shown in Fig. 4*B*. The initial potential recorded just after the impalement of a microelectrode was, sometimes, nearly equal to the resting potential level observed at control $[Ca]_o$ and the hyperpolarization of the resting potential followed (Fig. 4*A*), but, in some cases, such an initial level of resting potential instantaneously reached the hyperpolarized level on an impalement (Fig. 4*B*). These hyperpolarizations obtained at high $[Ca]_o$ might be induced by increases in the concentration of cytosol Ca^{2+} which activated the K^+ channel of cell membranes.

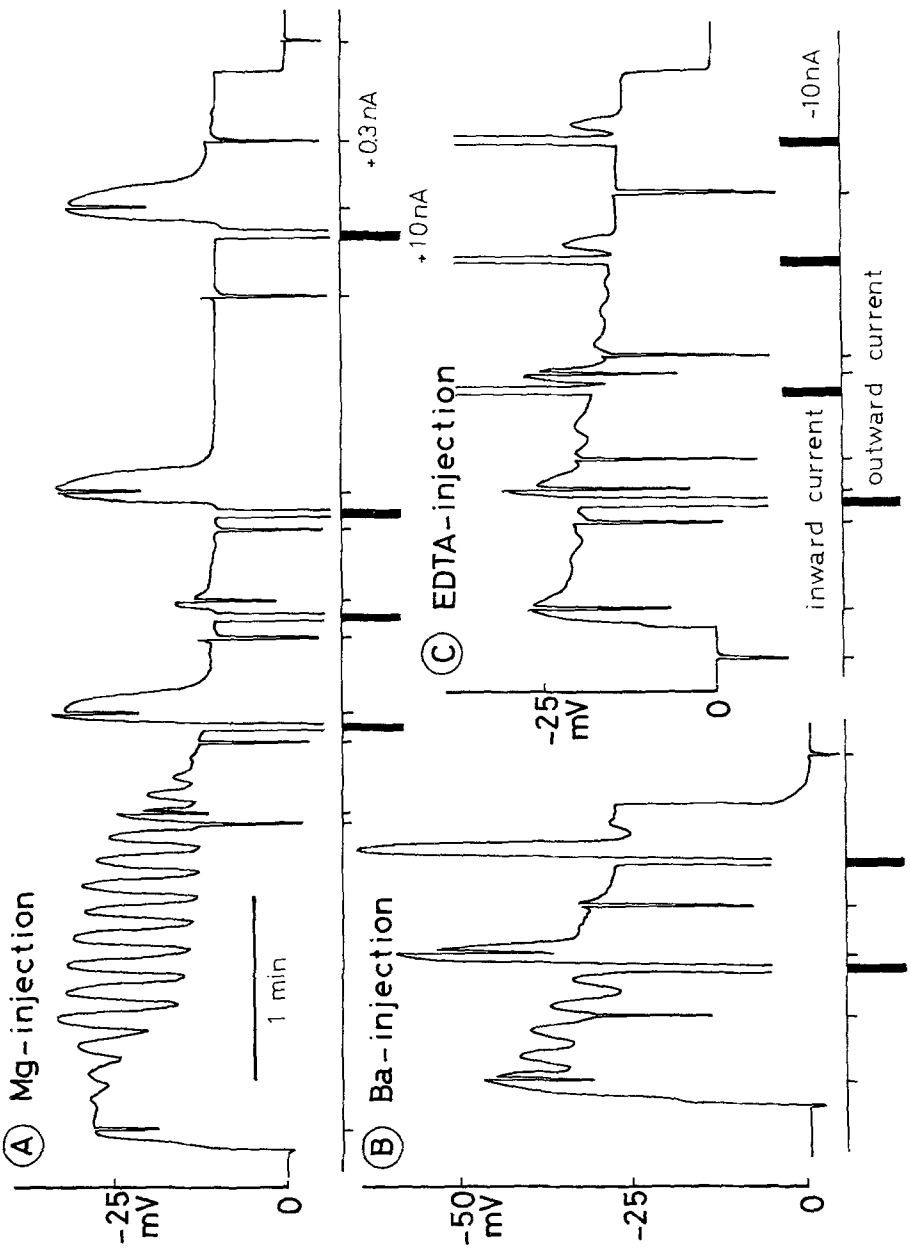

Fig. 3. Effects of intracellular Mg^{2+}, Ba^{2+} and EDTA injections (*A*, *B* and *C*, respectively). The divalent cations and anionic EDTA were injected with outward and inward currents of 10 nA, respectively, from microelectrodes filled with 3 M KCl as well as 0.1 M chloride salts of the cations and EDTA, respectively

A certain amount of Sr^{2+} or Mn^{2+} added to the external solution in place of Ca^{2+} was also able to induce the steady hyperpolarization of resting potential. Figure 4*C* shows plots of the steady hyperpolarizations of resting potentials thus induced by external Ca^{2+}, Sr^{2+} and Mn^{2+} against these external concentrations, and a simple manner of saturation is involved. In the absence of external Ca^{2+}, L cells showed

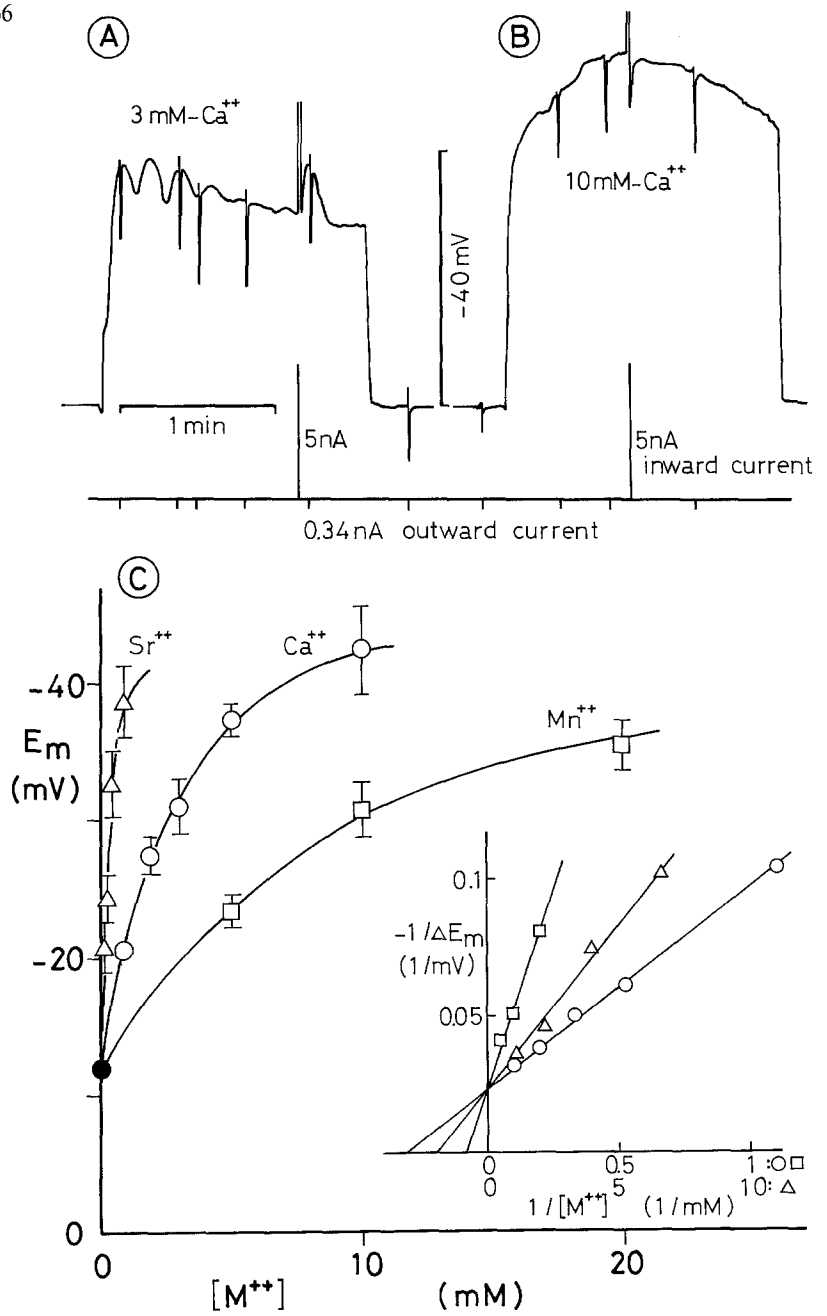

Fig. 4. Effects of Ca^{2+}, Sr^{2+}, and Mn^{2+} added to the external medium of Ca^{2+}-free TBS (in the presence of 0.5 mM-Mg^{2+}) on the membrane potential of L cells. (*A*): A typical chart of potential recording obtained in 3 mM-Ca^{2+} TBS. (*B*): in 10 mM-Ca^{2+} TBS. (*C*): Mean membrane potentials (E_m) were plotted against concentrations of divalent cations ([*M*]) applied to external media (Ca^{2+}, Sr^{2+} or Mn^{2+}). The E_m value of -10.9 mV obtained under Ca^{2+}-free conditions (Table 1) was used as the E_m value at $[M]=0$ mM. The vertical bars represent standard errors on either side of averages. *Insert:* A double reciprocal plot for the hyperpolarizing amplitude (ΔE_m) against [*M*]

spontaneous HRs and evoked HRs, when an appropriate amount of Sr^{2+} or Mn^{2+} was present in the external medium. Therefore, it is likely that Sr^{2+} as well as Mn^{2+} can enter the cell membrane and substitute for the intracellular Ca^{2+} not only for determining the level of resting potentials but also for generating HRs.

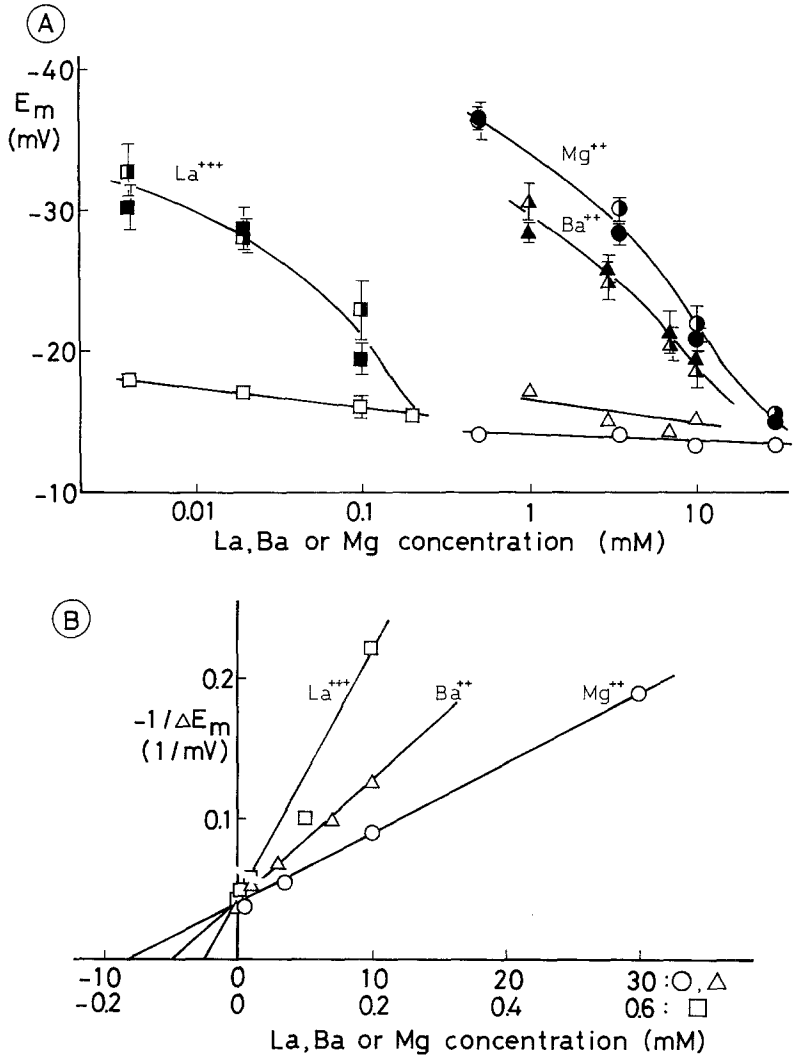

Fig. 5. Effects of external Mg^{2+}, Ba^{2+} and La^{3+} added to the bathing Mg^{2+}-free TBS in the presence of 0.9 mM Ca^{2+} on electrical properties of cell membranes. (*A*): Resting potentials (open symbols), spontaneous HRs (closed symbols) and evoked HRs (half-closed symbols) were plotted against external Mg^{2+}, Ba^{2+} and La^{3+} concentrations. The vertical bars represent standard errors on either side of averages. (*B*): Plot for the reciprocals of mean amplitudes of spontaneous and evoked HRs *vs.* external Mg^{2+}, Ba^{2+} and La^{3+} concentrations

In contrast, high doses of external Mg^{2+} and Ba^{2+} never induced hyperpolarizations of resting potentials, rather slight depolarizations were seen. Furthermore, these same doses suppressed both spontaneous and evoked HRs even in the presence of external Ca^{2+} (0.9 mM), as shown in Fig. 5*A*. The existence of a small amount of La^{3+} in the external solution also induced slight depolarizations of resting potentials and suppression of the HR activity (Fig. 5*A*). These effects of externally applied Mg^{2+} and Ba^{2+} are different from those of intracellularly injected Mg^{2+} and Ba^{2+}, because the latter preserved the HR evoked by an electrical stimulus. Moreover, the effect of external La^{3+} is opposite to that of intracellularly injected La^{3+}; the former depolarized the membrane and suppressed the hyperpolarizing response, whereas the latter produced marked hyperpolarization. In the light of these observations, it can be assumed that Mg^{2+}, Ba^{2+} and La^{3+} applied externally played a role in inhibiting the hyperpolarizing excitability on the outside surface of membrane, but not on the inside surface, presumably by competing for sites for the Ca^{2+} transport on the outer surface of membrane. This concept was supported by the following observation: ruthenium red, a well-known inhibitor of Ca^{2+}-binding to the binding site (glycoprotein moiety) of cell membrane (Kamino *et al.*, 1976), applied externally (2 mM) also inhibited not only oscillations of membrane potentials but also hyperpolarizing responses evoked by electrical stimuli (data not shown).

Some Characteristics of HRs and Hyperpolarizations Induced by High $[Ca]_o$

It has been shown that the hyperpolarizing responses of L cells are due to increases in K^+ conductance across the cell membrane (Okada *et al.*, 1977*b*; Roy & Okada, 1978), and the level of these HRs is linearly dependent on the logarithm of external K^+ concentration ($\log[K]_o$) (Okada *et al.*, 1977*b*). To examine such a $[K]_o$ dependency of hyperpolarizations of resting potentials induced by high $[Ca]_o$, the external K^+ concentrations were increased by the K^+-Na^+ replacement with high-$[Ca]_o$ TBS (10 mM Ca^{2+}). As shown in Fig. 6, the hyperpolarization level of resting potentials thus induced decreased with increases in $[K]_o$, and this $[K]_o$ dependency was quite similar to that of HR at 0.9 mM of $[Ca]_o$. This result strongly suggests that the hyperpolarization of resting potential induced by external high Ca^{2+} is also the result of an increase in the membrane K^+ conductance.

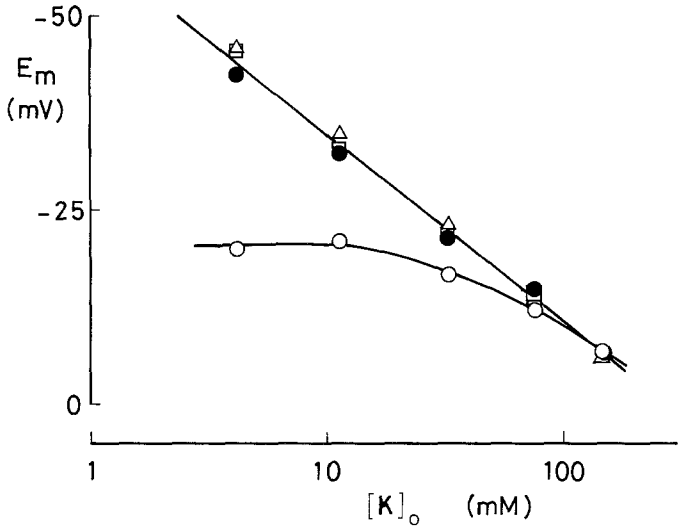

Fig. 6. Effects of $[K]_o$ changes produced by the KCl-NaCl substitution on resting potentials (○), spontaneous HRs (△) and evoked HRs (□) measured in 0.9 mM-Ca^{2+} TBS, and on resting potentials measured in a 10 mM-Ca^{2+} TBS (●). Each point represents the mean value of 6 ~ 19 observations with the standard error of less than ±3.4 mV

Perfusion of cells with a low-Na^+ solution (1 mM Na^+) caused a slight hyperpolarization of the resting potential when external Na^+ was replaced with $Tris^+$, as reported previously (Okada *et al.*, 1977*b*), but caused no change in the resting potential when external Na^+ was substituted by Li^+ (Table 3). In both low-Na^+ media, L cells produced continuous oscillations of membrane potentials and responded to electrical stimuli with hyperpolarizing responses. This result is in agreement with the concept that external Na^+ plays no essential role in the generation

Table 3. Effects of low $[Na]_o$ and high $[Ca]_o$ on membrane potentials of L cells

	0.9 mM Ca^{2+}		5 mM Ca^{2+}	
	Resting potential (mV)	Spontaneous HR (mV)	Resting potential (mV)	Spontaneous HR (mV)
Control (143 mM-Na^+)	−22.8±0.8 (27)	−40.7±1.8 (27)	−35.6±1.0 (17)	−44.4±1.6 (17)
1 mM-Na^+ (142 mM-Li^+)	−23.3±0.9 (12)	−39.8±2.7 (12)	−36.1±1.6 (14)	−44.3±2.4 (10)
1 mM-Na^+ (142 mM-$Tris^+$)	−32.2±0.6 (18)	−44.3±2.7 (18)	−36.5±1.6 (19)	−42.4±1.9 (11)

of these HRs in L cells (Okada *et al.*, 1977*b*). On application of high doses of external Ca^{2+} (5 mM), the cell membrane hyperpolarized in both low-Na^+ media. The amplitudes of such hyperpolarizations of resting potentials induced by high $[Ca]_o$ as well as of HRs observed in low-Na^+ media were almost the same as those observed in control TBS, as tabulated in Table 3. Therefore, it is hardly likely that a Na^+-Ca^{2+} exchange mechanism could participate in the generation of HR and hyperpolarization induced by high $[Ca]_o$. This is in good agreement with the report of no evidence for Na^+-Ca^{2+} exchange mechanism in L cells (Lamb & Lindsay, 1971).

At a low temperature (6 ± 2 °C), oscillations of membrane potentials were completely suppressed, and electrical stimuli rarely induced hyperpolarizing responses with a small amplitude as reported previously (Okada *et al.*, 1977*a*). In contrast to these dramatic disappearances of hyperpolarizing excitation, the external high Ca^{2+} (10 mM) continued to induce hyperpolarizations of resting potentials at a low temperature. The level of these hyperpolarizations (-40.4 ± 3.0 mV (20)) is not so different from that at $35 \sim 37$ °C (-43.4 ± 4.5 mV (10)). This result suggests that the hyperpolarization induced by high $[Ca]_o$ originates from a passive Ca^{2+} entry to the cell, but the hyperpolarizing response of the cell membrane may be related to an energy-dependent Ca^{2+} transport system.

Discussion

Deprivation of external Ca^{2+} produced depolarization of the resting potential and suppressed the hyperpolarizing excitability (i.e., spontaneous and evoked HRs) of L cells. External high Ca^{2+} concentrations made the resting potential hyperpolarized in a saturating manner, and the HR activity was maintained at high $[Ca]_o$ if the level of resting potentials was less hyperpolarized than that of HRs. These results indicate that the external existence of Ca^{2+} is indispensable for the generation of HR, and also that the external level of Ca^{2+} concentrations determines the level of resting potentials. It was concluded from the experiments with A23187 and Ca^{2+}-injection that these Ca ions are functioning on the inside of cell membranes due to stimulation of the Ca^{2+}-activated K^+ channel (or K^+ carrier) system which has been demonstrated in a variety of cell membranes (Whittam, 1968; Godfraind *et al.*, 1970, 1971; Lew, 1970; Meech & Strumwasser, 1970; van Rossum, 1970;

Blum & Hoffman, 1971, 1972; Riordan & Passow, 1971; Romero & Whittam, 1971; Feltz, Krnjević & Lisiewicz, 1972; Krnjević & Lisiewicz, 1972; Meech, 1972, 1974; Brown & Brown, 1973; Jansen & Nicholls, 1973; Gorman & Marmor, 1974; Gorman & McReynolds, 1974; Lassen *et al.*, 1974; Minota, 1974; Armando-Hardy *et al.*, 1975; Clusin, Spray & Bennett, 1975; Isenberg, 1975; Meech & Standen, 1975; Romero, 1976, 1978).

Assuming the cell membrane is at equilibrium and the individual ionic current across the membrane obeys a simple current-voltage relation, we can obtain the following equations for the membrane conductance (G_m) and potential (E_m).

$$G_m = \sum_i G_i \tag{1}$$

$$E_m = (\sum_i G_i E_i)/G_m \tag{2}$$

where i refers to each ionic species, G_i to the conductance, and E_i to the equilibrium potential of each ionic species. When the ionic conductance change is confined to K^+ (i.e., $\Delta G_m = \Delta G_K$), changes in the membrane potential (ΔE_m) can be expressed as follows.

$$-\Delta E_m = \frac{E_m^o - E_K}{G_m^o + 2\Delta G_K} \Delta G_K \tag{3}$$

where $G_m = G_m^o + \Delta G_m$, $E_m = E_m^o + \Delta E_m$, and G_m^o and E_m^o stand for the initial membrane conductance and potential, respectively. Eq. (3) predicts that the hyperpolarization induced by an increase in the K^+ conductance would, in a manner similar to Michaelis-Menten kinetics, increase with increasing ΔG_K. The plot of $(-1/\Delta E_m)$ *vs.* $(1/\Delta G_K)$ will give a straight line, the intercept on the abscissa representing the value of $[2/(E_m^o - E_K)]$.

Increases in the external Ca^{2+}, Sr^{2+} or Mn^{2+} concentration gave rise to a hyperpolarization of the cell membrane in a saturating manner due to stimulation of the Ca^{2+}-activated K^+ channel, as shown in Fig. 4*C*. The insert of this figure shows that the relation between the amplitude of hyperpolarizations ($-\Delta E_m$) and the concentration of these divalent cations obeyed the Michaelis-Menten type kinetics. In the light of this result, it could be inferred that the conductance change induced by the stimulation of the Ca^{2+}-activated K^+ channel is confined to K^+, as indicated by Blum & Hoffman (1971), assuming that ΔG_K is linearly dependent on the external concentration of Ca^{2+}, Sr^{2+} or Mn^{2+},

to a first approximation. Thus, the E_K value can be estimated from the intercept on the abscissa of this figure using the resting potential obtained in Ca^{2+}-free media (-10.9 mV; Table 1) as the E_m^o value. The E_K value thus estimated is around -98 mV, being in good accordance with those estimated from the intracellular K^+ concentration (-95.6 mV; Okada *et al.*, 1977*a*), from the reversal potential for the potential oscillation (-94 mV; Okada *et al.*, 1977*b*) and from the *I–V* curve for the K^+ current producing oscillations ($-85 \sim -90$ mV; Roy & Okada, 1978).

In light of all these experimental studies, it is reasonable to assume that an increase in the intracellular concentration of free Ca^{2+} actually takes place during the hyperpolarizing responses. As cooling the cell suppressed both spontaneous and evoked HRs, the Ca^{2+} transport system coupled with the HR activity would appear to be energy-dependent. In general, three cellular membranes are thought to contribute to the control of free calcium levels in the cytosol by transporting Ca^{2+} in an energy-dependent manner; namely, microsomal, mitochondrial, and plasma membranes. Indeed, an outward-directed active Ca^{2+} transport mechanism was characterized in the plasma membrane of L cells by Lamb and Lindsay (1971) and a microsomal Ca^{2+} transport activity in fibroblasts by Moore and Pastan (1977). The significant contribution of mitochondrial active Ca^{2+} transport to the regulation of cytosol Ca^{2+} concentrations was also demonstrated in cultured cells (Borle, 1972). It has been shown (Carafoli & Sottocasa, 1974) that the active Ca^{2+} transport system in mitochondria can also transport Sr^{2+} and Mn^{2+} with similarities to our results presented above. From the experimental observations of remarkable difference between effects of Mg^{2+}, Ba^{2+} and La^{3+} applied externally and those seen with internal application, it could be inferred that an increase in cytosol Ca^{2+} responsible for HR was the result of the Ca^{2+} entry from the external medium across the plasma membrane. This inference has been supported by our recent finding that verapamil, a Ca^{2+}-channel blocker, applied externally inhibits both evoked and spontaneous HRs in L cells (H. Yawo, Y. Okada & W. Tsuchiya, *unpublished observation*). In addition to a passive transport mechanism of Ca^{2+}, Sr^{2+} and Mn^{2+} which determines the resting potential level, therefore, the plasma membrane of L cell would have an energy-dependent, inwardly-directed Ca^{2+} transport mechanism which is responsible for the HR generation. Whether or not this inward Ca^{2+} transport mechanism is a reversed function of the outward active Ca^{2+} transport mechanism (Ca^{2+} pump) awaits further study for verification.

The external application of Mg^{2+}, Ba^{2+}, La^{3+} or ruthenium red inhibited both HRs in a dose-dependent manner (Fig. 5*A*). It has been shown in various cell membranes that magnesium ions compete for binding and transport sites with calcium ions (Rubin *et al.*, 1967; Foreman & Mongar, 1972) and lanthanum ions as well (Mela, 1968; Scarpa, Baldassare & Inesi, 1972; Weiss, 1974). In view of these facts, it is likely that the suppression of HRs by external Mg^{2+}, Ba^{2+} and La^{3+} is brought about by the competition of these cations with Ca^{2+} for the site of Ca^{2+} transport on the plasma membrane. Assuming that the Ca^{2+} transport is mediated by a carrier mechanism, a quantitative explanation of the dose dependency of the inhibiting effect of these cations (Fig. 5*B*) has been made (Okada *et al.*, 1978).

Under the experimental conditions employed herein, almost all the cells showed spontaneous repetitive hyperpolarizing responses, i.e., oscillations of membrane potential. Since a maintained high $[Ca]_i$ as a result of high $[Ca]_o$ or Ca^{2+} injection followed a sustained hyperpolarization, a continuously elevated $[Ca]_i$ does not seem to trigger an intermittent rise in G_K which would be expected from a refractory property of this K^+ channel after this temporary activation. Therefore, the periodic appearance of HRs may be explained in terms of a cyclic change in $[Ca]_i$. Similar periodic $[Ca]_i$ changes were actually observed in parallel to oscillations of the electrical potential in *Physarum* (Ridgway & Durham, 1976). Such oscillatory changes in the $[Ca]_i$ level could be brought about by a feed-back relationship between an energy-dependent, carrier-mediated Ca^{2+} entry mechanism and an active Ca^{2+} extruding pump on the plasma membrane of L cell.

The physiological function of such hyperpolarizing excitability has not been entirely elucidated. Recently, we found that cytochalasin B inhibited these hyperpolarizing responses of L cells concomitant with changes in cell morphology (Tsuchiya *et al.*, 1978; W. Tsuchiya, Y. Okada & A. Inouye (*in preparation*)). It has been shown that calcium plays a role in the regulation of movement in cultured fibroblasts by controlling the interaction between actin and myosin (Gail, Boone & Thompson, 1973; Izzard & Izzard, 1975). It is possible, therefore, that the $[Ca]_i$ increase accompanied with HR is related to cell motility. Though visible changes in cell morphology were not observed on each HR under phase microscopy (1,500 ×), we often noticed that the prolonged exposure of L cells to a high-Ca^{2+} medium made the cells round (i.e., possibly contracted), as was observed in mouse fibroblasts by Owens, Gey & Gey (1958). Gallin & Gallin (1977) found that hyperpolarizing responses were

induced by the application of some chemotactic factors (C5a, etc.) in macrophages. Such was not observed in L cells, but we did find that the membrane of the L cell can respond to some chemical stimuli (β-lipoprotein, crude compliment, etc.) with a sustained hyperpolarization (Tsuchiya *et al.*, 1978). There is, therefore, a possibility that the hyperpolarizing response of L cell is one kind of "receptor potential" associated with a cell motility system. Experiments are presently under way to further clarify the physiological meaning of the hyperpolarizing excitability of L cell, and detailed results will be presented in forthcoming reports.

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